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14. ABSTRACT The 14-3-3 proteins are a family of adaptor proteins that bind to more than 200 protein partners and affect their biological function by altering their stability, catalytic activity and subcellular localization. While the structure of 14-3-3 is well studied and the biology of the 14-3-3 interaction with their partners is understood, the regulation of these proteins remains debatable. Specifically, the mode of export of 14-3-3 proteins from the nucleus is not clear. A number of studies suggest a role for the CRM1 nuclear export receptor while other reports provide different findings. Here we describe a number of small molecules that affect the export of 14-3-3 sigma. One molecule, TK10, was previously shown to affect the transport of the transcription factor FKHR in a CRM1-independent manner. A second molecule, Haloprogin, an approved anti-fungal drug of unknown mechanism, was identified in a high-throughput screen of small molecules inhibitors of 14-3-3 export. Using the Rev-GFP reporter for nuclear export, we find that Haloprogin does not interfere with the CRM1 pathway. Haloprogin activity on different 14-3-3 isoforms is also studied. Therefore we determine that 14-3-3 sigma export from the nucleus is CRM1-independent. The results also suggest a mode of action for the drug Haloprogin.					
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INTRODUCTION

Complex signaling networks play a critical role in normal and tumor cell growth and their regulation is often dependent on compartmentalization of signal transducing and effector proteins within such networks. For instance, proteins such as transcription factors are held in the cytoplasm until the cell receives an external signal at which time the protein moves into the nucleus. Conversely, the export of proteins from the nucleus can be similarly regulated to control the nuclear presence of a particular protein such as a DNA or RNA binding protein, chaperones or kinases. This regulated movement acts as a robust readout for cellular responses to signals and provides a basis for potential therapeutic targets.

Central to many signaling pathways are the small 14-3-3 adaptor molecules that bind as dimers to phosphoserine and phosphothreonine [1-3] in a multitude of ligands, including oncoproteins and tumor suppressors [4-6] Figure 1. Such binding can alter the ligands' activity and subcellular localization [7-11]. Many of the seven 14-3-3 isoforms including 14-3-3 sigma modulate the activity and localization of their binding partners by shuttling between the nucleus and cytoplasm [12]. As a result, 14-3-3 proteins regulate cellular processes such as apoptosis, cell cycle and mitogenic signaling.

The regulation of 14-3-3 translocation is of interest because of its ability to bind a number of ligands that affect the regulation of tumor onset and development. For instance, the sigma isoform is absent in breast cancers [13], though apparently present in most other cancers. The localization of the protein seems to affect cellular fate as Huang et al. [14] found that 14-3-3 sigma was nuclear in prostate cancers and cytoplasmic in normal prostate tissue. Also, 14-3-3 sigma sequesters Bax and blocks apoptosis in adriamycin-treated cells, which would appear to be a pro-oncogenic activity. The sigma isoform is also of interest because it is induced by p53 in response to DNA damage [13]. Lack of 14-3-3 sigma results in an inability to maintain the G2/M checkpointing after treatment with a DNA-damaging agent, and cell death through mitotic catastrophe [13].

The mechanism by which 14-3-3 proteins, specifically the 14-3-3 sigma isoform, is exported from the nucleus into the cytoplasm remains unclear. It was initially thought that a putative NES within the 14-3-3 C-termini mediates the export of 14-3-3-ligand complexes [15]. However, recent findings question this "attachable NES model" and suggest that 14-3-3 proteins direct ligands out of the nucleus by exposing subcellular localization determinants within the bound cargo [16]. Early studies demonstrating 14-3-3 nuclear accumulation in response to treatment with leptomycin (LMB) [17-19] indicated that 14-3-3 export was dependent on the major nuclear export receptor CRM1 [16]. However, the effects of LMB on 14-3-3 nuclear export were observed at high LMB concentration following extended treatment periods [16]. In contrast, the nuclear export of proteins through the CRM1 receptor is usually inhibited by LMB at 1nM within 1 hour following treatment [20].

More recently, a number of groups showed that 14-3-3 sigma export is only marginally affected by LMB treatment [21, 22]. A different export receptor, Exportin 7, has been proposed as the primary nuclear export receptor for 14-3-3 sigma because 14-3-3 sigma binds Exportin 7 but not CRM1 in a Ran-GTP dependent manner [21].

In this study, we have first tested a number of small molecules previously identified as inhibitors of FKHR nuclear export [20]. FKHR is a downstream target of the PI3/Akt /PTEN signaling pathway (reviewed in [23]). Upon receiving growth-inducing cues, FKHR is phosphorylated on multiple sites by Akt. As a result, 14-3-3 sigma binds phospho-FKHR and promotes its export to the cytoplasm. The sequestration of FKHR in the cytoplasm

impairs FKHR ability to bind to DNA in the nucleus and prevents the transcription of target growth control and apoptotic genes.

The previously identified compound TK10 [20] which inhibits FKHR nuclear export also impedes the export of 14-3-3 sigma from the nucleus. Interestingly TK10 does not affect the export of proteins through CRM1, suggesting that 14-3-3 sigma export from the nucleus occurs at least in part in a CRM1-independent manner. We then developed a nuclear export assay and set up a high throughput screen to identify additional small molecules inhibitors of 14-3-3 sigma export. We were able to identify a number of such inhibitors, and we focus on the anti-fungal drug Haloprogin in this study. We find that Haloprogin impeded 14-3-3 nuclear export in a CRM1-independent fashion and that the compound affects different 14-3-3 isoforms. These compounds will be useful in exploiting alternative nuclear export mechanisms, understanding the biology of 14-3-3 sigma nuclear export, and might be further exploited for therapeutic purposes.

BODY

Effect of Leptomycin treatment on 14-3-3 nuclear export

The small molecule LMB has been shown to negatively affect the export of proteins from the nucleus through covalent binding and inhibition of the CRM1 nuclear export receptor [17-19]. It has been suggested that 14-3-3 export occurs through the CRM1 receptor and that treatment of mammalian cells with LMB results in the accumulation of 14-3-3 sigma in the nucleus [16]. We therefore treated HeLa cells that express 14-3-3 sigma with different concentration of LMB (1 nM, 10 nM and 20 nM) over a range of time (1, 2, 4, 12 and 24 hours) and immunostained the cells with the CS112A monoclonal antibody raised specifically against 14-3-3 sigma [16]. Our results (Figure 2a) show that accumulation of 14-3-3 sigma in the nucleus is minimal after 24 hours at the highest LMB concentration tested (20 nM) and that the majority of the protein remains cytoplasmic similar to the untreated cells (DMSO).

To ensure that this effect is not cell-type specific, we repeated the same experiment under similar conditions in U2OS cells that also express 14-3-3 sigma. We noted similar results (data not shown) to those observed in HeLa cells. We therefore concluded that while a small fraction of the 14-3-3 sigma proteins might be retained in the nucleus in response to LMB treatment, this compound on its own does not completely inhibit the nucleocytoplasmic transport of 14-3-3 sigma.

Effect of other CRM1 inhibitors on 14-3-3 nuclear export

Previously, our group had identified a number of small molecules that affect the nuclear export of the transcription factor FKHR [20, 24]. Upon further studies, it was determined that a subgroup of these compounds inhibit FKHR export from the nucleus through inhibition of the CRM1-dependent nuclear export pathway. Due to the overlap in the mode of activity of these compounds with LMB, we sought to examine the effect of these nuclear export inhibitors on the transport of 14-3-3 sigma from the nucleus into the cytoplasm; the rationale being that if 14-3-3 exits the nucleus via the CRM1 receptor pathway, these compounds might trap 14-3-3 in the nucleus. We therefore treated HeLa cells with the CRM1-dependent FKHR nuclear export inhibitors at the same concentrations that inhibit FKHR export (Figure 2b). While the compounds retained their ability to inhibit FKHR export (data not shown), none of the ones tested in this study showed an inhibitory effect on the export of 14-3-3 sigma (Figure 2b). This along with our LMB studies indicate that 14-3-3 sigma does not utilize the CRM1 export pathway to exit the nucleus and that molecules that affect this pathway cannot inhibit the export of 14-3-3 sigma from the nucleus.

Effect of non-CRM1 inhibitors on 14-3-3 sigma nuclear export.

A second subgroup of compounds identified by Kau et al. (2003) exhibit inhibitory activity on FKHR export in a CRM1-independent fashion. It was determined that these compounds modulate the activity of PI3K and calmodulin resulting in the retention of FKHR in the nucleus [20]. We sought to determine whether any of the CRM1-independent FKHR nuclear export inhibitors exhibit an effect on the nuclear translocation of 14-3-3 sigma into the cytoplasm. To this end, we treated HeLa cells with the CRM1-independent nuclear export inhibitors at concentrations similar to those that inhibit FKHR nuclear export. This

was followed by immunostaining of cells with 14-3-3 sigma specific antibodies. The results show that of the molecules assayed, treatment of cells with the compound TK10 at 40 μ M for 1 hour results in the accumulation of 14-3-3 sigma in the nucleus (Figure 2c). This result supports the proposal that 14-3-3 sigma translocation across the nuclear envelope utilizes a CRM1-independent pathway.

Screening for small molecules inhibitors of 14-3-3 sigma nuclear export.

Based on the results presented above, we hypothesized that the export of 14-3-3 sigma occurs in a CRM1-independent manner. We therefore sought to identify additional small molecules that exhibit a regulatory effect on the transport of 14-3-3 sigma from the nucleus into the cytoplasm through screening of a large collection of small molecules.

We have therefore developed a high-content high-throughput cell-based assay to identify small molecules which when added to cells result in the retention of 14-3-3 sigma in the nucleus. The screen was performed in HeLa cells in 384-well plates compatible for high-throughput pin transfer of library compounds. Approximately 30,000 compounds originating from diverse small molecule commercial and public libraries were screened. From the original screen, 7 compounds were identified that reproducibly result in the nuclear accumulation of 14-3-3 sigma. Upon initial characterization of these compounds, we determined that 4 exhibit intrinsic fluorescence properties and have a nuclear subcellular localization, and therefore the signal detected in the nucleus following treatment with these compounds was not specific to 14-3-3 sigma (data not shown). These were dropped from further analysis. Of the remaining three compounds identified from the 14-3-3 nuclear export screen we focused on the characterization of Haloprogin [25, 26] (Figure 3a and 3b) as it showed the most pronounced effect on 14-3-3 nuclear accumulation in comparison with TK10 and the other two newly identified molecules.

Effect of Haloprogin treatment on 14-3-3 nuclear export

We first sought to determine whether the effect of Haloprogin on 14-3-3 nuclear export is cell-type specific. For this we treated both HeLa and U2OS cells with 40 μ M of Haloprogin for 1 hour (similar conditions to the ones used in the screen). We then performed immunostaining using the CS112A 14-3-3 sigma specific antibodies. The data shows that Haloprogin inhibits the export of 14-3-3 sigma from the nucleus in both cell types, suggesting that the effect is not specific to HeLa cells. We then determined the minimal conditions necessary for Haloprogin activity on 14-3-3 sigma transport in order to minimize cytotoxicity. For this, we studied the effect of Haloprogin treatment on 14-3-3 nuclear export at concentrations ranging from 5 to 80 μ M for periods of 5 to 90 minutes (Figure 4 and data not shown). As shown by our results, Haloprogin inhibition of 14-3-3 sigma reaches saturation at 40 μ M following 30 minute treatments. Lower doses of Haloprogin (5 μ M) have no effect on 14-3-3 sigma, while at 10-20 μ M Haloprogin inhibitory effect is partial. We also studied the inhibition of 14-3-3 sigma with Haloprogin and Leptomycin B side by side and determined that Haloprogin but not Leptomycin B inhibit the nuclear export of the protein (Figure 5)

Haloprogin does not affect the CRM1-dependent export pathway

We assayed the ability of Haloprogin to inhibit the export of proteins that are known to exit the nucleus through binding to CRM1. For this we overexpressed Rev-GFP fusion protein in HeLa cells. Under normal conditions Rev-GFP proteins shuttle in and out of the nucleus and the steady state level of the protein is cytoplasmic. In contrast, treatment of HeLa cells expressing Rev-GFP with LMB at 10 nM for 1 hour results in the accumulation of the otherwise cytoplasmic Rev-GFP in the nucleus. We treated the HeLa-Rev-GFP cell line with DMSO, LMB (10 nM) or Haloprogin (40 μ M) for 1 hour and analyzed the subcellular localization of Rev-GFP under each condition. The results show that while LMB treatments result in the accumulation of Rev-GFP in the nucleus, treatment of cells with Haloprogin does not alter the cytoplasmic localization of Rev-GFP similar to treatment with DMSO. We assayed the subcellular localization of 14-3-3 sigma in these cells under the same conditions and found that Haloprogin retains its ability to inhibit 14-3-3 sigma nuclear export while LMB effects are comparable to those observed in DMSO treated cells. We therefore conclude that similar to TK10, Haloprogin inhibits the export of 14-3-3 sigma from the nucleus in a CRM1-independent fashion while the CRM1 inhibitor LMB does not affect the export of 14-3-3- sigma.

Specificity of Haloprogin towards different 14-3-3 isoforms

In order to determine whether the effect of Haloprogin observed is specific to 14-3-3 sigma isoform, we assayed the subcellular localization of other 14-3-3 isoforms following treatment HeLa cells with Haloprogin. Specifically, we studied the subcellular localization of the gamma, tau, beta and beta/zeta isoforms to whom antibodies are commercially available and which express detectable levels of proteins in HeLa cells. We found (Figure 6) that Haloprogin is not specific to regulating the export of 14-3-3 sigma isoform. Treatment of cells with Haloprogin results in the accumulation of the beta and tau isoforms but not the gamma isoform in the nucleus (Figure 6). In studies that looked at the beta/zeta isoforms, we noted that Haloprogin results in the accumulation of proteins in the nucleus, however the signal from proteins in the cytoplasm remained significant (Figure 6). This might suggest that Haloprogin affects the zeta isoform translocation.

KEY RESEARCH ACCOMPLISHMENTS

Effect of TK10 on 14-3-3 export

- TK10 an inhibitor of FKHR export from the nucleus inhibits 14-3-3 sigma nuclear export. Studies to determine dose-dependent response and IC50.
- In the same cell line, TK10 inhibits 14-3-3 sigma export but does not affect the transport of Rev-GFP through the CRM1 receptor.

Screen of compounds inhibitors of 14-3-3 sigma nuclear export

- Screen of nuclear export inhibitors is fully automated and the methodology can be used for further visual cell-based screens of small molecules as well as for overexpression screens (miRNA, siRNA, cDNA, and others).
- Screened to date more than 50,000 small molecules.
- Identification of a number of hits and focus on Haloprogin.
- Possible mode of function for Haloprogin which was previously unknown.

Characterization of Haloprogin

- Haloprogin inhibits 14-3-3 sigma nuclear export in a cell-type-independent manner
- The drug inhibits 14-3-3 nuclear export in a dose dependent manner
- The drug shows different activity towards different 14-3-3 isoforms
- The drug does not affect nuclear export through the CRM1 receptor.
-

Genomic screen of microRNA library for 14-3-3 sigma nuclear export inhibitors

- Conditions determined for optimal transfection of miRNA. About 80% transfection efficiency achieved.
- Screening of ~300 microRNAs
- Identification of multiple hits that inhibit the export of 14-3-3 sigma
- Combine siRNA and miRNA data to try and determine targets of miRNA

REPORTABLE OUTCOMES

Presentations

- Poster presentation at Systems Biology Retreat, Harvard Medical School
- Six Group Meeting presentation, Dr. Pamela Silver laboratory
- Manuscript in preparation for publication
- Presentations at job interviews

Funding applied for based on the work supported by this training grant

- NIH funding for Dr. Pamela Silver

Research opportunities applied for and/or received based on experience/training supported by this grant

- Collaboration with Novartis Institutes for Biomedical Research to screen a library of siRNA that targets the whole genome to identify inhibitors of 14-3-3 nuclear export. The collaboration started as a small project to screen the kinase collection and have recently expanded to include the whole genome.
- Collaboration with Ambion, Inc. Research and Development group to screen an expanded version of microRNA collection for inhibitors of 14-3-3 nuclear export. The collaboration started with a subset of the miRNA library and have since expanded to all the available miRNAs.
- Multiple job interviews for scientific positions

CONCLUSIONS

We have used a chemical biology approach to study the nuclear export of 14-3-3 sigma in mammalian cells to further understand the regulation of its nucleocytoplasmic translocation. We find that the small molecule LMB only marginally inhibit the export of 14-3-3 sigma from the nucleus suggesting that its export is not CRM1-dependent in line with recent reports [21]. A number of other small molecules, namely TK10 and Haloprogin, are newly identified robust inhibitors of 14-3-3 sigma export. Interestingly, these molecules do not inhibit the CRM1-dependent pathway for protein translocation into the cytoplasm as shown by their lack of effect on the export of Rev-GFP proteins. Whether 14-3-3 sigma affects the Exportin 7 [21] pathway in mammalian cells remains to be seen. Our preliminary studies (data not shown) show that the protein levels of Exportin 7 are not altered in response to Haloprogin or TK10 treatment at concentrations that inhibit 14-3-3 nuclear export.

TK10 was previously identified in a screen for inhibitors of FKHR nuclear export [20]. TK10 was shown to exert inhibitory activity of FKHR export by regulating the PI3K/Akt pathway [20]. Addition of TK10 results in accumulation of the hypo-phosphorylated form of TK10 in the nucleus. While the target pathway of TK10 is identified, the biology of Haloprogin is not well understood. Haloprogin, generic for 1,2,4-trichloro-5-(3-iodoprop-2-ynoxy)benzene, is an antifungal chemical typically used in the treatment of fungal (Tinea) skin such as athlete's foot, jock itch, ringworm and tinea versicolor, infections caused by dermatophytes (Trichophyton, Microsporum and Epidermophyton). The mechanism of action of the drug is unknown however it is speculated that Haloprogin exhibits antifungal activity by interfering with the yeast membrane structure and function [25, 26]. Since Haloprogin inhibits the export of 14-3-3 sigma from the nucleus, this hints to a model for the mode of action of Haloprogin in its anti-fungal effect. It remains to be tested whether Haloprogin exhibit its anti-fungal effect through interfering with the export of proteins from the nucleus.

Finally, the role 14-3-3 sigma plays in the onset and development of tumors make it a therapeutic target candidate. For instance, a number of conventional anti-cancer drugs are selective for rapidly dividing cells and their efficiency is reduced in apoptosis-resistant cells [27]. Therefore, disruption of 14-3-3 activity by blocking its nuclear export or ligand binding will possibly enhance the effect of anti-cancer drugs. In this respect, inhibition of 14-3-3-ligand binding by short peptides increased cell sensitivity to apoptosis upon treatment with lower than conventional doses of cisplatin [28]. The emerging role of 14-3-3 in the regulation of cell response to DNA damage [15, 29-32] makes it a valid target for cancer therapy. As a number of therapeutics function as DNA damaging agents, disruption of the G2/M checkpoint renders cells more vulnerable to cell death as this prevents cell cycle arrest to repair damaged DNA. In this respect, studies on the UCN-01 compound that inhibits the G2/M checkpoint by inhibiting Cdc25C phosphorylation on the 14-3-3 binding site are encouraging [28, 33]. Consequently, small molecules that disrupt the activity of 14-3-3 will potentially exhibit synergistic effects when used in combination with DNA damaging regimens (e.g. chemotherapy and γ -irradiation).

REFERENCES

1. Yaffe MB, Rittinger K, Volinia S, Caron PR, Aitken A, Leffers H, Gambelin SJ, Smerdon SJ, Cantley LC: **The structural basis for 14-3-3:phosphopeptide binding specificity.** *Cell* 1997, **91**(7):961-971.
2. Wilker EW, Grant RA, Artim SC, Yaffe MB: **A structural basis for 14-3-3sigma functional specificity.** *J Biol Chem* 2005, **280**(19):18891-18898.
3. Yaffe MB, Elia AE: **Phosphoserine/threonine-binding domains.** *Curr Opin Cell Biol* 2001, **13**(2):131-138.
4. Kumagai A, Yakowec PS, Dunphy WG: **14-3-3 proteins act as negative regulators of the mitotic inducer Cdc25 in Xenopus egg extracts.** *Mol Biol Cell* 1998, **9**(2):345-354.
5. Jiang K, Pereira E, Maxfield M, Russell B, Godelock DM, Sanchez Y: **Regulation of Chk1 includes chromatin association and 14-3-3 binding following phosphorylation on Ser-345.** *J Biol Chem* 2003, **278**(27):25207-25217.
6. Zha J, Harada H, Yang E, Jockel J, Korsmeyer SJ: **Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-X(L).** *Cell* 1996, **87**(4):619-628.
7. Fujita N, Sato S, Katayama K, Tsuruo T: **Akt-dependent phosphorylation of p27Kip1 promotes binding to 14-3-3 and cytoplasmic localization.** *J Biol Chem* 2002, **277**(32):28706-28713.
8. Fujita N, Sato S, Tsuruo T: **Phosphorylation of p27Kip1 at threonine 198 by p90 ribosomal protein S6 kinases promotes its binding to 14-3-3 and cytoplasmic localization.** *J Biol Chem* 2003, **278**(49):49254-49260.
9. Davezac N, Baldin V, Gabrielli B, Forrest A, Theis-Febvre N, Yashida M, Ducommun B: **Regulation of CDC25B phosphatases subcellular localization.** *Oncogene* 2000, **19**(18):2179-2185.
10. Giles N, Forrest A, Gabrielli B: **14-3-3 acts as an intramolecular bridge to regulate cdc25B localization and activity.** *J Biol Chem* 2003, **278**(31):28580-28587.
11. Dalal SN, Schweitzer CM, Gan J, DeCaprio JA: **Cytoplasmic localization of human cdc25C during interphase requires an intact 14-3-3 binding site.** *Mol Cell Biol* 1999, **19**(6):4465-4479.
12. Tzivion G, Avruch J: **14-3-3 proteins: active cofactors in cellular regulation by serine/threonine phosphorylation.** *J Biol Chem* 2002, **277**(5):3061-3064.
13. Dubois T, Rommel C, Howell S, Steinhussen U, Soneji Y, Morrice N, Moelling K, Aitken A: **14-3-3 is phosphorylated by casein kinase I on residue 233. Phosphorylation at this site in vivo regulates Raf/14-3-3 interaction.** *J Biol Chem* 1997, **272**(46):28882-28888.
14. Huang D, Liu X, Plymate SR, Idowu M, Grimes M, Best AM, McKinney JL, Ware JL: **Proteomic identification of 14-3-3 sigma as a common component of the androgen receptor and the epidermal growth factor receptor signaling pathways of the human prostate epithelial cell line M12.** *Oncogene* 2004, **23**(41):6881-6889.
15. Lopez-Girona A, Furnari B, Mondesert O, Russell P: **Nuclear localization of Cdc25 is regulated by DNA damage and a 14-3-3 protein.** *Nature* 1999, **397**(6715):172-175.
16. Brunet A, Kanai F, Stehn J, Xu J, Sarbassova D, Frangioni JV, Dalal SN, DeCaprio JA, Greenberg ME, Yaffe MB: **14-3-3 transits to the nucleus and participates in dynamic nucleocytoplasmic transport.** *J Cell Biol* 2002, **156**(5):817-828.
17. Fukuda M, Asano S, Nakamura T, Adachi M, Yoshida M, Yanagida M, Nishida E: **CRM1 is responsible for intracellular transport mediated by the nuclear export signal.** *Nature* 1997, **390**(6657):308-311.
18. Kudo N, Wolff B, Sekimoto T, Schreiner EP, Yoneda Y, Yanagida M, Horinouchi S, Yoshida M: **Leptomycin B inhibition of signal-mediated nuclear export by direct binding to CRM1.** *Exp Cell Res* 1998, **242**(2):540-547.

19. Kudo N, Matsumori N, Taoka H, Fujiwara D, Schreiner EP, Wolff B, Yoshida M, Horinouchi S: **Leptomycin B inactivates CRM1/exportin 1 by covalent modification at a cysteine residue in the central conserved region.** *Proc Natl Acad Sci U S A* 1999, **96**(16):9112-9117.
20. Kau TR, Schroeder F, Ramaswamy S, Wojciechowski CL, Zhao JJ, Roberts TM, Clardy J, Sellers WR, Silver PA: **A chemical genetic screen identifies inhibitors of regulated nuclear export of a Forkhead transcription factor in PTEN-deficient tumor cells.** *Cancer Cell* 2003, **4**(6):463-476.
21. Mingot JM, Bohnsack MT, Jakle U, Gorlich D: **Exportin 7 defines a novel general nuclear export pathway.** *Embo J* 2004, **23**(16):3227-3236.
22. van Hemert MJ, Niemantsverdriet M, Schmidt T, Backendorf C, Spaink HP: **Isoform-specific differences in rapid nucleocytoplasmic shuttling cause distinct subcellular distributions of 14-3-3 sigma and 14-3-3 zeta.** *J Cell Sci* 2004, **117**(Pt 8):1411-1420.
23. Burgering BM, Kops GJ: **Cell cycle and death control: long live Forkheads.** *Trends Biochem Sci* 2002, **27**(7):352-360.
24. Schroeder FC, Kau TR, Silver PA, Clardy J: **The psammaplysenes, specific inhibitors of FOXO1a nuclear export.** *J Nat Prod* 2005, **68**(4):574-576.
25. Rezabek GH, Friedman AD: **Superficial fungal infections of the skin. Diagnosis and current treatment recommendations.** *Drugs* 1992, **43**(5):674-682.
26. Rudolph RI: **Haloprogin as treatment for fungal infections.** *Clin Exp Dermatol* 1979, **4**(4):548.
27. Cao W, Chi WH, Wang J, Tang JJ, Lu YJ: **TNF-alpha promotes Doxorubicin-induced cell apoptosis and anti-cancer effect through downregulation of p21 in p53-deficient tumor cells.** *Biochem Biophys Res Commun* 2005, **330**(4):1034-1040.
28. Bunch RT, Eastman A: **Enhancement of cisplatin-induced cytotoxicity by 7-hydroxystaurosporine (UCN-01), a new G2-checkpoint inhibitor.** *Clin Cancer Res* 1996, **2**(5):791-797.
29. Chan TA, Hermeking H, Lengauer C, Kinzler KW, Vogelstein B: **14-3-3Sigma is required to prevent mitotic catastrophe after DNA damage.** *Nature* 1999, **401**(6753):616-620.
30. Chen L, Liu TH, Walworth NC: **Association of Chk1 with 14-3-3 proteins is stimulated by DNA damage.** *Genes Dev* 1999, **13**(6):675-685.
31. Wilker E, Yaffe MB: **14-3-3 Proteins--a focus on cancer and human disease.** *J Mol Cell Cardiol* 2004, **37**(3):633-642.
32. Yarden RI, Pardo-Reoyo S, Sgagias M, Cowan KH, Brody LC: **BRCA1 regulates the G2/M checkpoint by activating Chk1 kinase upon DNA damage.** *Nat Genet* 2002, **30**(3):285-289.
33. Shao RG, Cao CX, Shimizu T, O'Connor PM, Kohn KW, Pommier Y: **Abrogation of an S-phase checkpoint and potentiation of camptothecin cytotoxicity by 7-hydroxystaurosporine (UCN-01) in human cancer cell lines, possibly influenced by p53 function.** *Cancer Res* 1997, **57**(18):4029-4035.

SUPPLEMENTAL MATERIALS

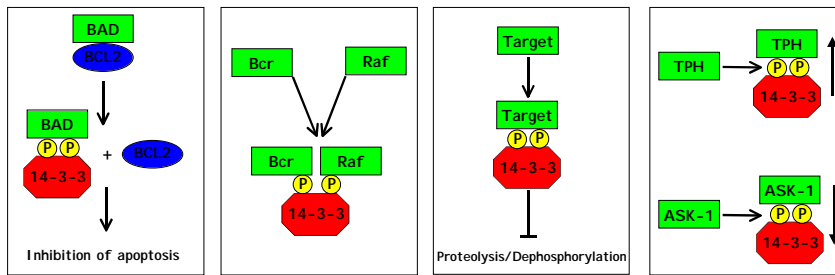


Figure 1. 14-3-3 functions and targets. In addition to modulating nuclear import and export rates, 14-3-3 proteins can: 1) promote dissociation of protein complexes by sequestering a phosphorylated binding partner; 2) promote interaction between phosphorylated proteins, such as between Bcr and Raf; 3) block the proteolysis or dephosphorylation of a target protein; and 4) modulate the enzymatic activity of phosphorylated proteins.

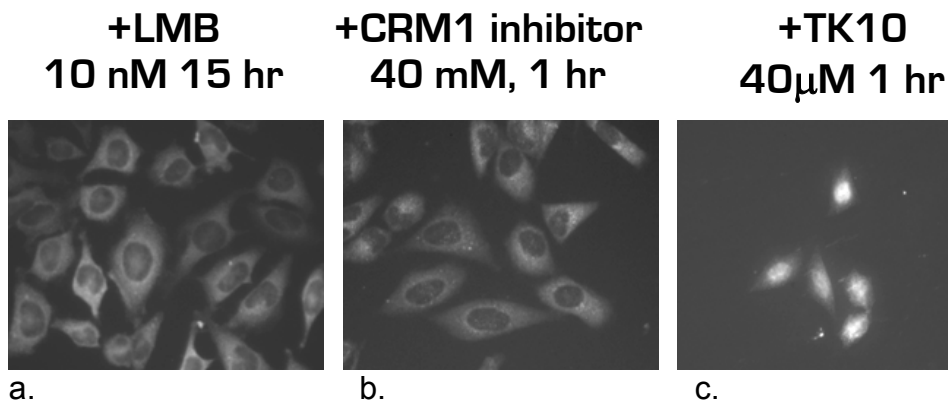
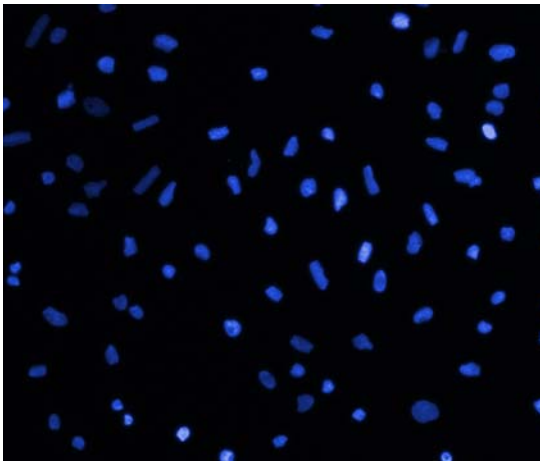
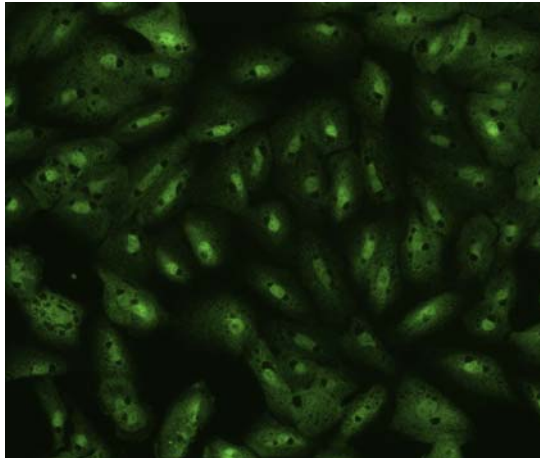
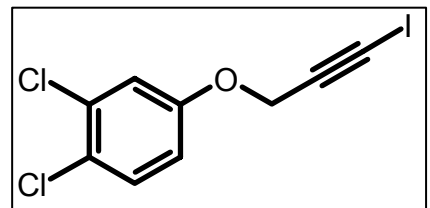


Figure 2. Subcellular localization of 14-3-3 sigma in mammalian cells in response to treatment with the CRM1 inhibitor Leptomycin B (a), other CRM1 inhibitor (b) and CRM-1 independent nuclear export inhibitor TK10 (c).

Haloprogin 40 μ M



a.



b.

Figure 3. Relocalization of 14-3-3 sigma to the nucleus(a, top) of HeLa cells upon treatment with Haloprogin. The nuclear staining overlaps with staining of nuclei with DAPI (a, bottom). Chemical structure of Haloprogin.

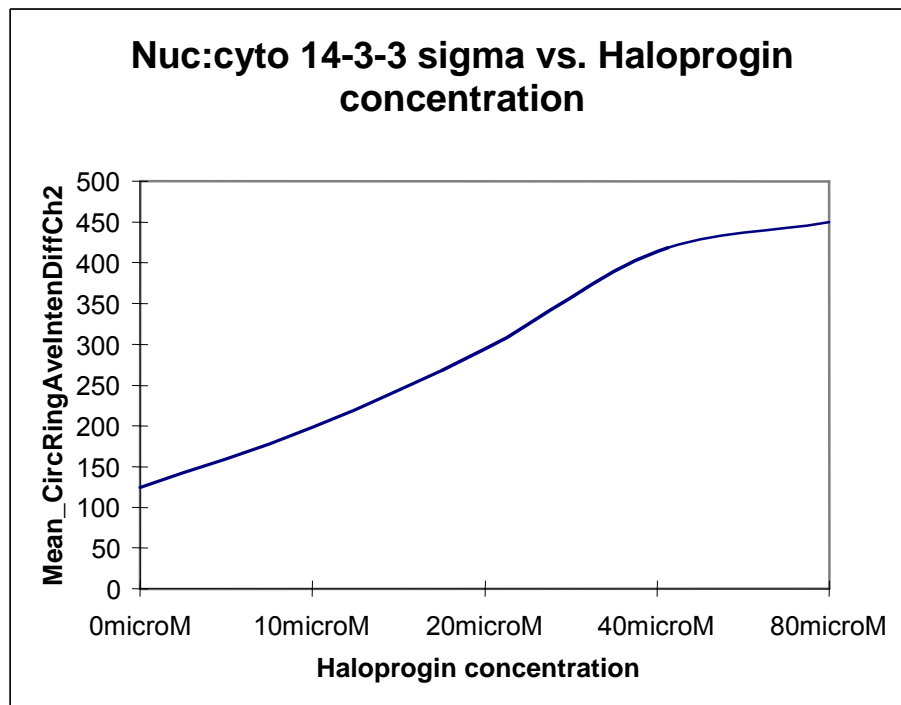


Figure 4. Dose dependent inhibition of 14-3-3 sigma nuclear export. Y axis reflects signal intensity in the nucleus versus cytoplasm..

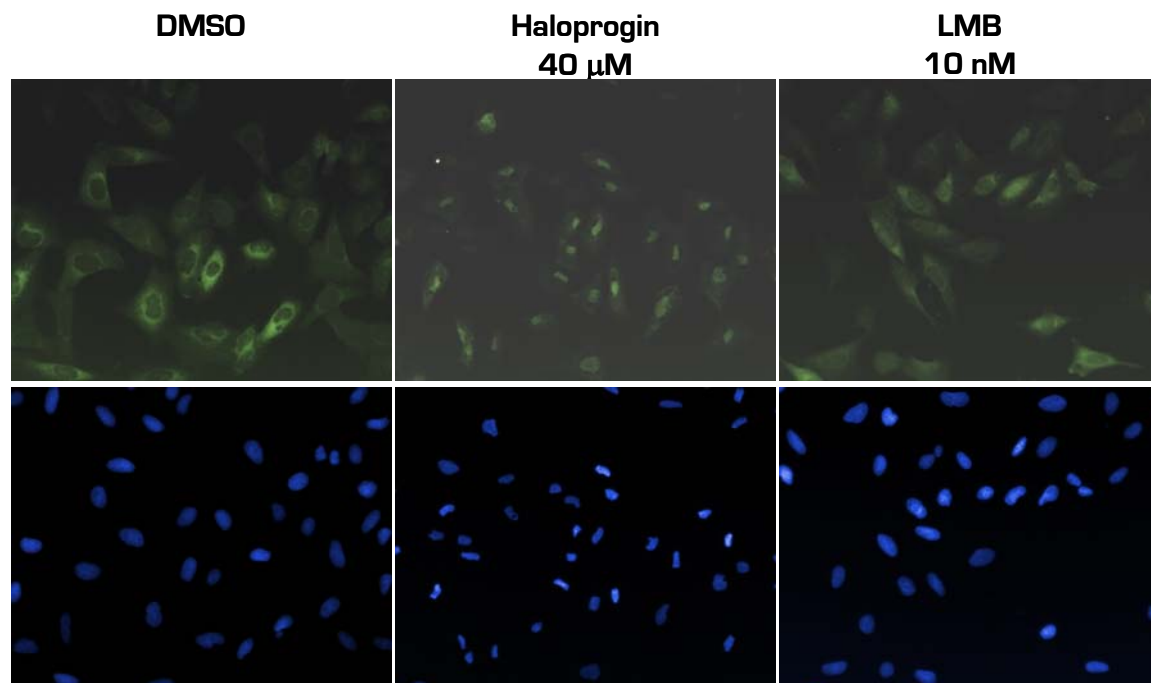


Figure 5. Side by side study of the effect of Haloproglin and LMB on 14-3-3 sigma nuclear export.

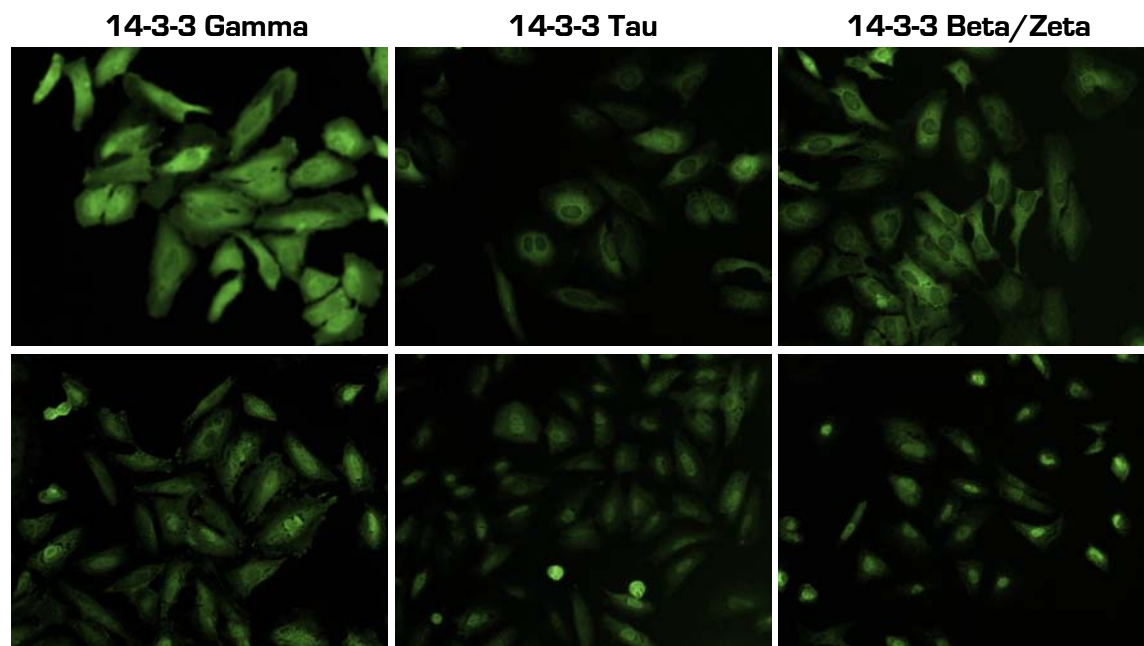


Figure 6. Effect of Haloproginin on the export of different 14-3-3 isoforms from the nucleus. Top panel is distribution of isoforms in the cell prior to treatment with Haloproginin. Bottom panel represents the effect of treatment with Haloproginin.